이학박사 학위논문

CALHM family 단백질간의 상호작용과 세포내 칼슘 조절 메커니즘에 관한 연구

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지도교수 서 유 헌

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하성지의 박사학위논문을 인준함

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A study on the interactions among the CALHM family proteins and their mechanisms of intracellular Ca^{2+} regulation

By

Ha Sung Ji

A thesis submitted in partial fulfillment of the requirements for the
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Doctoral Committee

Professor _____ **Chairman**

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Professor _____

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ABSTRACT

A study on the interactions among the CALHM family proteins and their mechanisms of intracellular Ca^{2+} regulation

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Science to the faculty of Interdisciplinary Program in Brain Science

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Calcium (Ca^{2+}) plays several fundamental roles in regulating diverse neural processes such as membrane excitability and synaptic transmission, and perturbed calcium homeostasis in neurons are implicated in neurodegenerative diseases including Alzheimer's disease (AD). The calcium homeostasis modulator 1(CALHM1) is a leaky Ca^{2+} channel that is localized mainly at the endoplasmic reticulum (ER) and plasma membrane (PM) and is known to modulate the intracellular Ca^{2+} signals. The single nucleotide polymorphism (SNP) of the CALHM1-P86L (rs2986017) was reported to be associated with AD yet this association is still controversial.

There are two other CALHM family proteins, CALHM2 and CALHM3; however their functional properties remain unknown. As CALHM family proteins share sequence homology and similar structures, we hypothesized that CALHM family subtypes interact with each other and subsequently their interaction affects their function.

Here, we showed that CALHM2 and CALHM3 are localized at the ER membrane and form novel Ca^{2+} channels through mutual interaction as well as their expression in the mouse brain. We found that although CALHM1 and CALHM3 are leaky Ca^{2+} channels, CALHM2 is inactive by itself and CALHM2 regulates CALHM1 and CALHM3 channel activities when constitutes heteromeric channels with them. These results suggest that CALHM family members may exist as heteromeric Ca^{2+} channels in the brain and contribute to the regulation of intracellular Ca^{2+} homeostasis

Key words: Calcium homeostasis, CALHM (Calcium homeostasis modulator) family, Alzheimer's disease, Ca^{2+} channel

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LIST OF ABBREVIATION

AD, Alzheimer’s disease

ANOVA, analysis of variance

APP, amyloid precursor protein

Aβ, amyloid beta peptide

Ca^{2+}, calcium

CALHM, calcium homeostasis modulator

cDNA, complementary DNA

CNBr, cyanogen bromide

CPA, cyclopiazonic acid

DAPI, 4',6-diamidino-2-phenylindole

ER, endoplasmic reticulum

FAM26, family with sequence similarity 26

FBS, fetal bovine serum

GFP, green fluorescent protein

GST, glutathione S-transferase

HBSS, HEPES-buffered salt solution

IgG, Immunoglobulin G

LSD, least significant difference

MBP, maltose binding protein

mRNA, messenger RNA

NMDA, N-Methyl-D-aspartic acid

PBS(T), phosphate buffered saline (Triton X-100)

PCR, polymerase chain reaction

PM, plasma membrane

PS, presenilin

RT, room temperature

RT-PCR, reverse transcription polymerase chain reaction

SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase

SDS-PAGE,sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNP, single nucleotide polymorphism

TM, transmembrane

UPR, unfolded protein response

Δ, delta

INTRODUCTION

Calcium (Ca^{2+}) is implicated in diverse neural processes such as membrane excitability, synaptic transmission, gene expression, and cell death and is important for executing higher brain functions. Therefore, perturbation in neuronal Ca^{2+} homeostasis is thought to cause diverse neurodegenerative diseases including Alzheimer's disease (AD) (Bezprozvanny and Mattson 2008; Marambaud, Dreses-Werringloer et al. 2009; Berridge 2010). Many studies have shown that AD causing mutations of amyloid precursor protein (APP) and presenilins (PS) are associated with perturbed intracellular Ca^{2+} signaling, which is caused by dysregulation of various Ca^{2+} channels and pumps on the plasma membrane (PM) and endoplasmic reticulum (ER) membrane (LaFerla 2002; Smith, Green et al. 2005; Supnet and Bezprozvanny 2010).

Recently, calcium homeostasis modulator 1 (CALHM1, previously annotated as FAM26C), a Ca^{2+} permeable channel, was reported in influencing Ca^{2+} homeostasis, Aβ levels, and AD risks (Dreses-Werringloer, Lambert et al. 2008; Boada, Antunez et al. 2010; Cui, Zheng et al. 2010).

CALHM1 is a multipass transmembrane glycoprotein and that is localized mainly at the ER membrane and partially at the PM. Overexpression of CALHM1 decreases extracellular Aβ accumulation and elevates sAPPα level in APP-transfected mouse neuroblastoma N2a cells (Dreses-Werringloer, Lambert et al. 2008). In addition, CALHM1 increases intracellular and mitochondrial Ca^{2+} concentration and reduces ER Ca^{2+}-contents by decreasing both the transport capacity and the Ca^{2+} affinity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which causes ER stress (Dreses-Werringloer, Lambert et al. 2008; Moreno-Ortega, Ruiz-Nuno et al. 2010; Gallego-Sandin, Alonso et al. 2011).

It has been suggested that the P86L, single nucleotide polymorphism (SNP) of the CALHM1 is associated with AD (Dreses-Werringloer, Lambert et al. 2008; Boada, Antunez et al. 2010; Cui, Zheng et al. 2010) but other studies failed to find such association (Inoue, Tanaka et al. 2010; Shibata, Kuerban et al. 2010; Feher, Juhasz et al. 2011). Although the role of CALHM1 in AD is controversial, CALHM1-P86L shows impaired channel activity of CALHM1 (Dreses-Werringloer, Lambert et al. 2008; Moreno-Ortega, Ruiz-Nuno et al.

2010).

Besides CALHM1, there are two other members among the CALHM family proteins, CALHM2 (FAM26B) and CALHM3 (FAM26A). However, little is known about them. One Japanese group studied about the SNPs in all genes of the CALHM family and reported no detectable association of the SNPs of the three genes with AD (Shibata, Kuerban et al. 2010). The expression of CALHM family proteins in TRPM5-positive taste cells was also reported, although their physiological functions in taste signaling were unknown (Moyer, Hevezi et al. 2009).

CALHM1 has sequence similarities with the selectivity filter of the NMDA receptor (Dreses-Werringloer, Lambert et al. 2008). NMDA receptors have several subtypes that have differences in sensitivity to ligands, permeation and blockage by divalent ions and kinetics and their functional properties are determined by subunit composition (Cull-Candy, Brickley et al. 2001), Waxman and Lynch 2005). In addition, CALHM2 and CALHM3 share sequence homology with CALHM1. Thus we hypothesized that CALHM subtypes interact with each other and their functions might be modulated by

mutual interaction, similar to the actions of NMDA receptor.

In the present study, we first examined functional properties of CALMH2 and CALHM3 as Ca^{2+} channels. We also examined whether CALHM subtypes constituted of heteromeric channels that modulate the functional properties of the channels. Our results demonstrate that CALHM3 is a leaky Ca^{2+} channel as similar to CALHM1, whereas CALHM2 is not permeable for Ca^{2+} by itself. In addition, we found that CALHM subtypes interact with each other and that their mutual interaction modulates the functional properties of the channels. Our findings suggest that CALHM family members may exist as heteromeric Ca^{2+} channels to modulate Ca^{2+} homeostasis in the brain.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the RIKEN guidelines for animal experiments.

Cell culture and transfection

HeLa cells and HEK293T cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). All cell lines were cultured in Dulbecco's modified Eagle's medium (Nakarai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Nakarai Tesque). Transfections of DNAs were performed using FuGENE HD (Promega, WI, USA) according to the manufacturer's instruction.

Plasmid DNA

Human CALHM1 cDNAs in the pcDNA3.1 Myc-His vector were generously

recived from Dr. Philippe Marambaud. These cDNAs restricted with EcoRI and XhoI and transferred into pEGFP-N1 vector (Clontech, CA, USA). Human CALHM2 and CALHM3 were amplified by PCR using the primers 5'-GGCTCGAGATGGCAGCCCTGATCGCAGAG-3'and 5'- CCGAATTC CGGAGGGGAGCAGGGCCATCTC-3' for CALHM2 and 5'-GGAAGCTT ATGGATAAATTCCGATGCTC-3' and 5'-CCGAATTCCCACGTCGGTG TGTTGTGACAG-3' for CALHM3 (the underlined letters indicate restriction enzyme site) and a HeLa cell cDNA as a template. The PCR fragments were digested by appropriate restriction enzymes and cloned into pEGFP-N1 and pcDNA3.1 Myc-His C vector (Invitrogen, NY, USA).

RT-PCR

The specific primers used were: mouse CALHM1: CCGAATTC CTGGAAC TGGGTCATACCCACGG,GGCTCGAGTTAGCATTTGTGCCAGCTTGTG AG; mouse CALHM2: CCGAATTCAAGGATGACGAAGAGCTGGTCGC

C, GGCTCGAGTTAAGCAGTGAGCAGGGCCATCTCCACATTG; mouse CALHM3: CCGAATTCCGTGCCCTGGGGTTACGTCGGGAC, GGCTCGAGTTAGCTGGAGTACCAGGTACTGAGGAGTTG; GAPDH: ATACGGCTACAGCAACAGGGTG, CTCTCTTGCTCAGTGTCCTTG. The reaction used was: 30 sec at 94˚C, 30 sec for 63˚C, 20 sec at 68˚ C for 40 cycles. Ten ng of cDNA from the individual parts of the mouse brain were used as a template. The amplified PCR fragments were cloned (underlines in the primer sequences were sequence for restriction enzyme digestion) and the sequence was confirmed.

Antibodies

GST fusion proteins with the COOH-terminal region of each CALHM family protein were produced in E.coli and used as antigens. Rabbits were immunized with these antigens and complete Freund's adjuvant by subcutaneous injection at 14-day intervals. All antibodies were affinity-purified by passing the immunized rabbit's serum through the CNBr-activated

Sepharose 4B (GE Health care AB, Sweden) column covalently coupled with MBP-CALHM family protein. Other antibodies used were: mouse anti-Myc antibody (9E10) (Santa Cruz Biotechnology Inc., CA, USA)), rabbit anti-Calreticulin antibody (abcam, Tokyo, Japan), mouse anti-GFP antibody (MBL International Corporation, Nagoya, Japan), Alexa 594-conjugated goat anti-rabbit IgG (Invitrogen, CA, USA) and Alexa 488-conjugated goat anti-mouse IgG (Invitrogen, CA, USA).

Immunocytochemistry

Transfected cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and blocked with 1% skim milk with 1% normal goat serum (Vector Laboratories, CA, USA) in PBS for 1h at RT. Then they were incubated with primary antibody for 1h at RT, washed with PBS three times and incubated with secondary antibody for 1h at RT. After washing with PBS three times, the coverslips were mounted with Vectashield containing 4’,6-diamidino-2-phenylindole (DAPI, Vector

Laboratories) and observed under a confocal fluorescence microscope (FV1000, Olympus, Tokyo, Japan).

Co-immunoprecipitation

HEK293T cells expressing CALHM family were solubilized in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and proteinase inhibitors). The homogenates were centrifuged at 15,000 rpm for 10 min. Supernatants were incubated with 3 g of primary antibody and 30 μl of 50% protein G-Sepharose 4B Fast Flow (GE Healthcare AB, Sweden) for overnight at 4℃. The immuno-complexes were washed three times with lysis buffer and proteins were eluted by SDS polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.01% bromophenol blue).

Western Blotting

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham Pharmacia, Buckinghamshire, United Kingdom). The membrane was blocked with 5% skim milk in PBST for 1h at RT and incubated with primary antibody for 1h at RT. After incubation, membrane were washed with PBST three times and incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia) for 1h at RT. After washed with PBST, immunoreactive bands on the membrane were visualized with Immobilon Western Detection Reagents (Millipore, MA, USA) and LAS-4000 mini (Fuji Film, Tokyo, Japan).

Ca^{2+} imaging

Intracellular Ca^{2+} imaging was performed as described previously (Higo, Hattori et al. 2005). After 12h of transfection, cells were loaded with 5 μM fura-2 AM (Dojindo, Japan) for 30 min at RT. Then, the cells were placed on the stage of an inverted microscope (IX-70; Olympus, Japan) and perfused

with HEPES-buffered salt solution (HBSS) (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, pH 7.4, 10 mM glucose and 2 mM $CaCl_2$). Image was captured and processed with an Argus 50/CA system (Hamamatsu Photonics, Japan) at RT. Delta R (ΔR) is the change in the ratio (F340/F380) of the fluorescent intensities of fura-2AM that are excited by the light at a wavelength of 340 and 380 nm. For determining the amount of Ca^{2+} store size, cells were treated 10 μM cyclopiazonic acid (CPA; Sigma-Aldrich, Rehovot, Israel) under Ca^{2+} free HBSS (Bergner, Kellner et al. 2009). Before the imaging, GFP fluorescence images were acquired to identify the transfected cells. The Ca^{2+}-increasing rate was calculated by amount of $[Ca^{2+}]_i$ change divided by time (min).

Statistical Analysis

Statistical analysis was done using SPSS (Chicago, IL). Data were expressed as mean ± S.E. values. Student's *t*-test or One-way ANOVA followed by Tukey's test and Least Significant Difference (LSD) test was used for determining statistical significance.

RESULTS

CALHM subtypes share sequence homology and secondary structures.

Typically, function of new proteins can be inferred from other proteins with similar sequences, since their common origin is assumed to be reflected in their structure and function (Abascal and Valencia 2003). For the protein structure and function prediction, we analyzed amino acid sequences of human CALHM family proteins using a multiple sequence alignment (Corpet 1988). As shown in Figure 1, all CALHM family proteins showed relatively high consensus sequence (red: high (90%) consensus, blue: low (50%) consensus) and CALHM2 and CALHM3 showed about 44% and 22% identities with CALHM1.

The predicted secondary structures are shown in Figure 2. CALHM2 has three transmembrane (TM) domains and CALHM3 has four TM domains similar to CALHM1. With these sequence homology and similar structures, we speculated that other CALHM family subtypes may also function as a Ca^{2+} channel like CALHM1.

CALHM subtypes bind to each other and constitute heteromeric channels.

Although the expressions of CALHM1 at the ER and the PM were previously reported (Dreses-Werringloer, Lambert et al. 2008), there are no information for the intracellular localizations of CALHM2 and CALHM3. To examine the intracellular localization of each CALHM family proteins in cells, we first exogenously expressed each subtypes of CALHM family protein in HeLa cells and performed immunocytochemistry. We found that CALHM1 was mainly localized at the ER and some of the signals were also observed at or near the PM as previously reported (Dreses-Werringloer, Lambert et al. 2008) (Fig. 3A). We also found that CALHM2 and CALHM3 showed similar reticular expression patterns much like CALHM1 when expressed alone and were extensively co-localized with the ER resident protein, calreticulin (Figure 3A). To examine whether CALHM subtypes colocalized with each other in cells, we then expressed two subtypes of CALHM in HeLa cells and examined their intracellular localization. We found that the particular reticular expression pattern was also observed in the cells co-expressing two types of

CALHM family proteins and the two CALHM family protein signals were extensively co-localized with each other (Figure 3B).

We next performed the co-immunoprecipitation assay using HEK293T cells transiently expressing two subtypes of CALHM family proteins to see if CALHM family proteins formed heteromeric channels. When we overexpressed CALHM1-myc and CALHM2-GFP and immunoprecipitated CALMH1-myc with anti-CALHM1 antibody, CALHM2-GFP was co-immunoprecipitated as shown in Figure 3C. CALHM2 was not detected in the control anti-rabbit-IgG immunoprecipitates (Figure 3C). When we co-transfected with CALHM2-myc and CALHM3-GFP and immunoprecipitated CALHM2-myc with anti-CALHM2 antibody, CALHM3-GFP was imunoprecipiated (Figure 3D). Interaction between CALHM1 with CALHM3 was also confirmed in cells overexpressing CALHM3-myc and CALHM1-GFP (Figure 3E). Similar results were obtained in HeLa cells transiently expressing CALHM family proteins (data not shown). These results suggested that CALHM family proteins heteromulitimerized at the ER and PM *in vitro*.

CALHM family subtypes are expressed in the brain.

Whereas CALHM1 mRNA expression in the mouse brain was previously demonstrated (Dreses-Werringloer, Lambert et al. 2008), and the expression of CALHM2 and CALHM3 mRNA remains unknown. To examine the mRNA expressions of CALHM2 and CALHM3 in the brain, we performed RT-PCR with specific primers for each CALHM subtypes using cDNAs from various parts of the mouse brain as a template. We examined each subtypes of CALHM mRNA in cortex, hippocampus, striatum, and cerebellum, and confirmed the expression of all subtypes of CALHM mRNA in those brain regions (Figure 4). Thus, the result suggested that all CALHM subtypes are expressed in the brain.

CALHM1 and CALHM3, but not CALHM2, form Ca^{2+} leaky homomeric channels.

Although CALHM1 channel was shown to be a constitutively open Ca^{2+} channel at the PM and also worked as an ER Ca^{2+} leak channel (Moreno-Ortega, Ruiz-Nuno et al. 2010; Gallego-Sandin, Alonso et al. 2011), the nature of CALHM2 and CALHM3 channel as a Ca^{2+} channel was still

unknown. To examine whether CALHM2 and CALHM3 could have Ca^{2+} channel properties by themselves, we overexpressed each CALHM family protein in HeLa cells and examined intracellular Ca^{2+} signals within the cells. Whereas only gradual elevation of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was observed in the control cells when we elevated extracellular Ca^{2+} concentration from 0 (nominally free) to 2 mM, the robust Ca^{2+} entry was detected in the GFP-tagged CALHM1 overexpressing cells in response to the stimulation as previously reported (Figure 5A).

CALHM3-GFP overexpression also significantly increased Ca^{2+} entry as compared to the control cells, although the ratio change of $[Ca^{2+}]_i$ was smaller than that of GFP-tagged CAHLM1 (Figure 5A and 5B). In contrast, GFP-tagged CALHM2 expression did not significantly affect Ca^{2+} entry as compared to the control cells (Figure 5A-5C). The Ca^{2+}-increasing rates were increased by about 70-fold and 2-fold in CALHM1 and CALHM3 expressing cells, respectively (Figure 5B). The ratio change of $[Ca^{2+}]_i$ at the steady state also increased by 15-fold and 4-fold as compared control cells in CALHM1 and CALHM3 expressing cells (Figure 5C). These results suggested that both

CALHM1 and CALHM3 were Ca^{2+} leaky channels, whereas CALHM2 could not function as a Ca^{2+} permeable channel by itself.

Overexpression of CALHM1 and CALHM3 decreases Ca^{2+} content within the ER.

It is known that overexpression of CALHM1 decreases Ca^{2+} concentration within the ER by increasing the rate of Ca^{2+} leaking from the ER and by reducing the ER Ca^{2+} uptake through the SERCA (Gallego-Sandin, Alonso et al. 2011). Therefore, we tested whether CALHM2 and CLAHM3 expression could affect ER Ca^{2+}-content. In order to measure the ER Ca^{2+}-content, we treated CALHM family transfected cells with 10 μM cyclopiazonic acid (CPA), an inhibitor for SERCA, in the nominal absence of external Ca^{2+} (Bergner, Kellner et al. 2009). As shown in Figure 6A, the CPA treatment induced a passive Ca^{2+} efflux from the ER and increased the $[Ca^{2+}]_i$ in the cells. We calculated the area under curve (AUC) of the $[Ca^{2+}]_i$ to estimate the Ca^{2+}-contents of the ER for each CALHM family transfected cells (Figure 6B). As shown in Figure 6B, the Ca^{2+}-contents of the ER of cells overexpressing

CALHM1 and CALHM3 were about 6-fold and 2-fold less than those of the control cells, respectively. In contrast, CALHM2 expression didn't significantly affect the ER Ca^{2+}-store size (Figure 6B).

CALHM2 modified Ca^{2+} channel properties of CALHM1 and CALHM3.

Since CALHM2 did not show apparent Ca^{2+} channel activity by itself and interacted with CALHM1 and CALHM3 (Figure 3 and Figure 4), we next examined whether CALHM2 could modulate the Ca^{2+} permeability of CALHM1 and CALHM3. We monitored $[Ca^{2+}]_i$ of the HeLa cells transfected with GFP-tagged CALHM1 only or co-transfected with Myc-tagged CALHM2 in response to change of extracellular Ca^{2+} concentration from 0 to 2 mM. We measured Ca^{2+} signals of the cells expressing GFP signals as similar levels between single and co-transfected conditions. Interestingly, we found that the increasing rate of $[Ca^{2+}]_i$ in the CALHM1 and CALHM2 co-transfected cells was 3 times slower than that in CALHM1 singly transfected cells: 4.43 ± 0.41 for CALHM1 and 1.41 ± 0.25 for CALHM1 and CALHM2

co-transfected cells (mean ± S.E.) (Figure 7A and 7C).

The maximal $[Ca^{2+}]_i$ was decreased by 2-fold in CALHM1 and CALHM2 co-expressing cells than those in CALHM1 single expressing cells (Figure 7D). We also examined the Ca^{2+}-content within the ER of the doubly transfected cells. Although the Ca^{2+}-content within the ER tended to be slightly increased in CALHM1 and CALHM2 co-expressing cells compared to CALHM1 expressing cells, however statistical significance could not be determined between them (Figure 7E). On the other hand, co-expression of CALHM2 with CALHM3 slightly but significantly increased the $[Ca^{2+}]_i$ -increasing rate by 2-fold and increased the maximal $[Ca^{2+}]_i$ by 1.5-fold compared to CALHM3 single expression (Figure 7B and 7C). Curiously, the Ca^{2+}-content of the ER was also significantly increased by CALHM2 co-expression with CALHM3. These results suggest that CALHM2 interacts with CALHM1 and CALHM3 and regulates their channel activities in the opposite way.

CALHM1-P86L shows impaired channel activity and that activity is modulated by CALHM2.

In order to see the effect of CALHM1 mutation (P86L) on Ca^{2+} regulation, we transfected HeLa cells with CALHM1-P86L and measured $[Ca^{2+}]_i$. To induce the Ca^{2+} influx from the PM, we transiently depleted external Ca^{2+} and monitored $[Ca^{2+}]_i$ under HBSS containing 2 mM $CaCl_2$. CALHM1-P86L expression showed significantly increased Ca^{2+} influx compared to the control cells (Figure 8B). The Ca^{2+}-increasing rate and maximal $[Ca^{2+}]_i$ at the steady state of CALHM1-P86L expressing cells were significantly increased (Figure 8C and 8D) and ER Ca^{2+}-content was decreased compared to the control cells (Figure 8E). However, these channel activities were decreased compared to CALHM1-WT as previously reported (Dreses-Werringloer, Lambert et al. 2008; Moreno-Ortega, Ruiz-Nuno et al. 2010) (Figure 5 and Figure 8)

We confirmed interaction between CALHM1-P86L and CALHM2 using co-immunoprecipitation assay (Figure 8A) and then examined weather CALHM2 also regulates channel activity of CALHM1-P86L. CALHM2 co-expression with CALHM1-P86L decreased Ca^{2+}-increasing rate from

0.355±0.14 to 0.99±0.12 (about 3-fold) and lowered the $[Ca^{2+}]_i$ at the steady state from 0.52±0.04 to 0.25±0.30 (2-fold) compared to CALHM1-P86L single expression (Figure 8C and 8D). We also measured ER Ca^{2+}-content and found that CALHM2 co-expression with CALHM1-P86L reduced ER Ca^{2+}-content compared to CALHM1-P86L single expressing cells (Figure 8E). These results indicated that CALHM-P86L decreased channel activity of CALHM1-WT and that the activity was regulated by CALHM2.

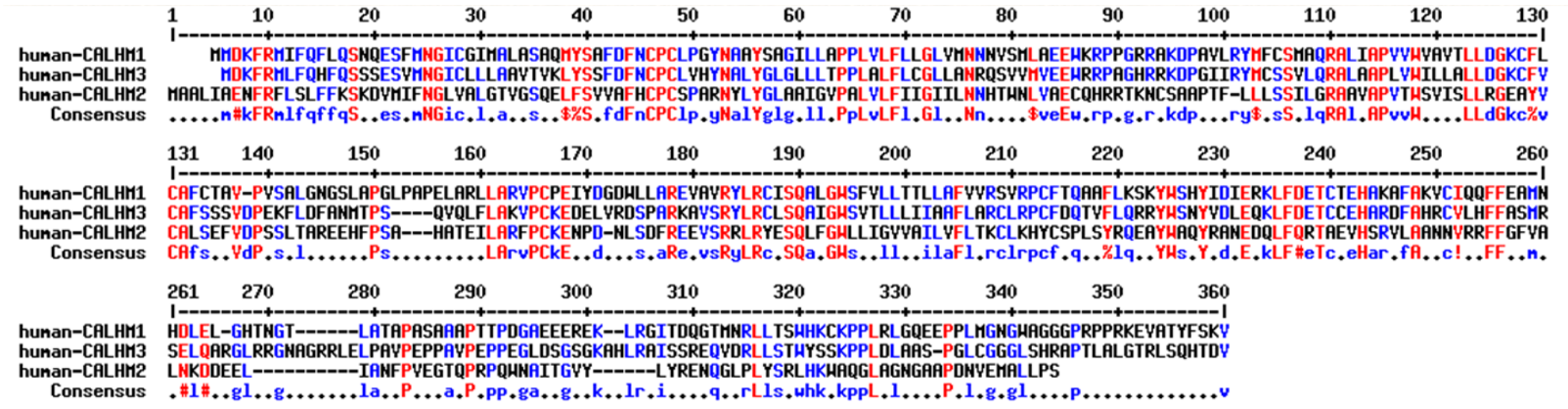

Figure 1. CALHM family proteins share sequence homology.

Using Multalin ver 5.4.1, amino acid sequences of CALHM family proteins were compared with each other.

(High consensus: Red, Low consensus : Blue)

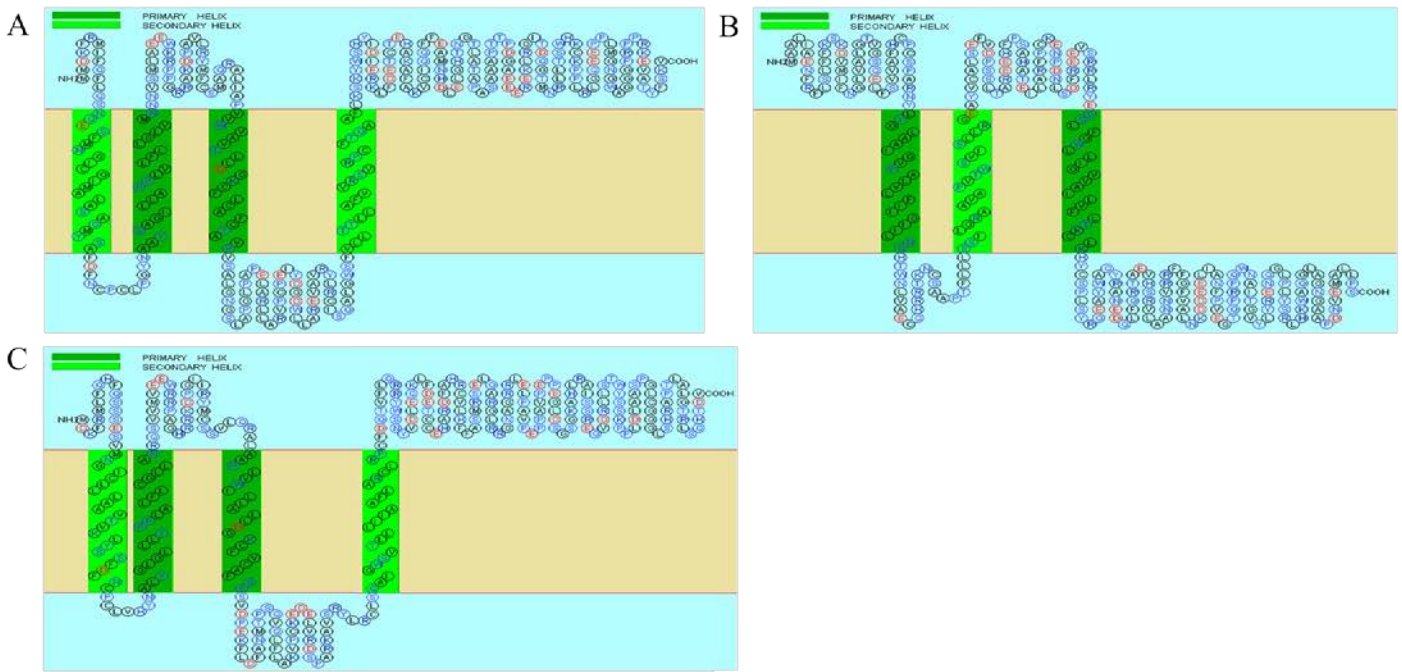

Figure 2. CALHM family proteins have similar secondary structures.

Predicted secondary structures of CALHM family subtypes. (A) CALHM1, (B) CALHM2 and (C) CALHM3 (By SOSUI system)

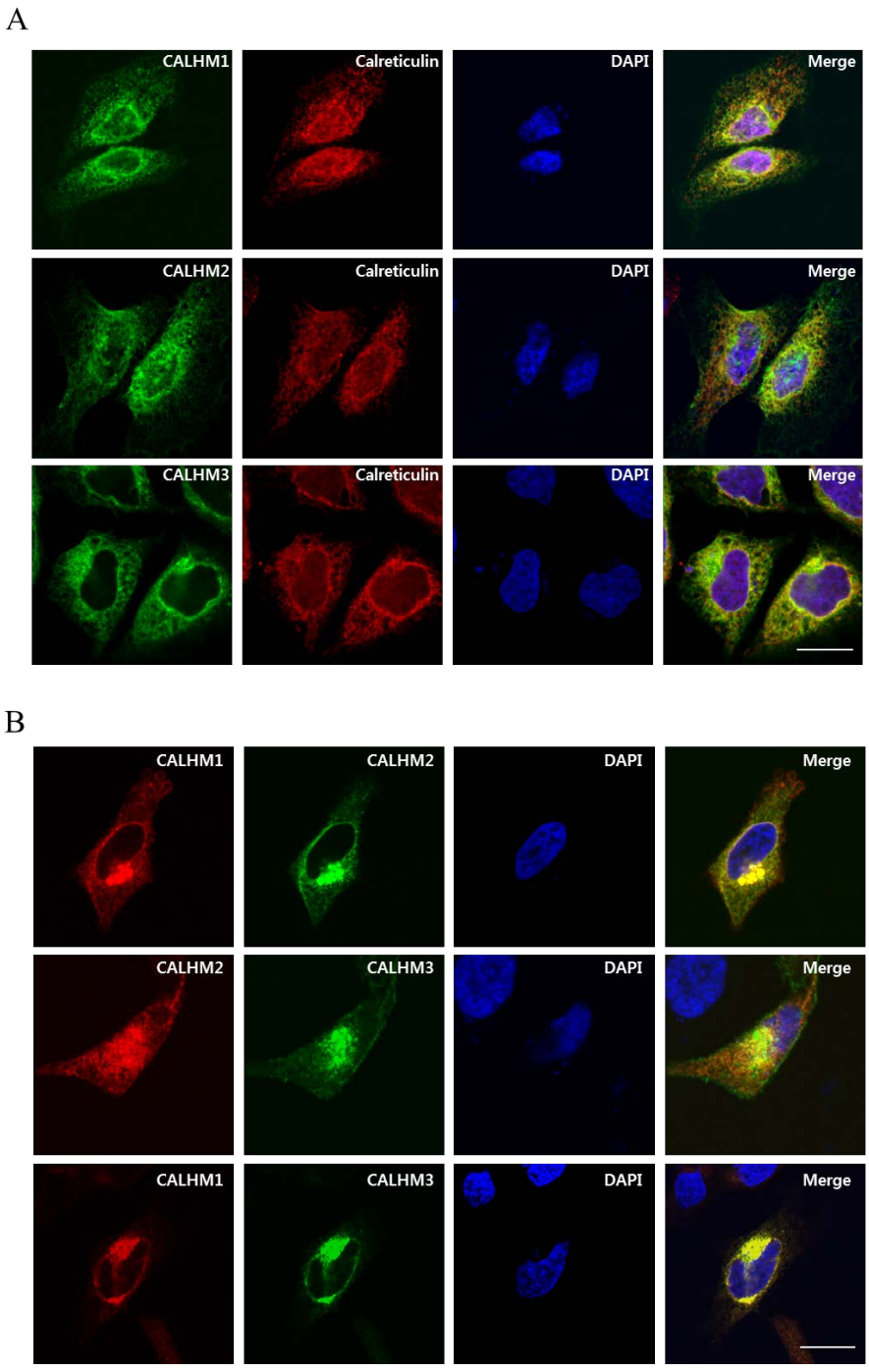

Figure 3. CALHM family interacts with each other.

(A) Cellular localizations of CALHM family proteins in HeLa cells that were singly transfected with GFP tagged-CALHM family proteins, respectively. Cells were stained with anti-GFP (green), anti-Calreticulin (red) and DAPI (blue). Scale bar, 20 μm (B) CALHM family proteins colocalize with each other in HeLa cells. Cells overexpressing GFP-tagged CALHM2 or CALHM3 (green) and myc-tagged CALHM1 or CALHM2 stained with anti-Myc antibody (red). Scale bar, 20 μm.

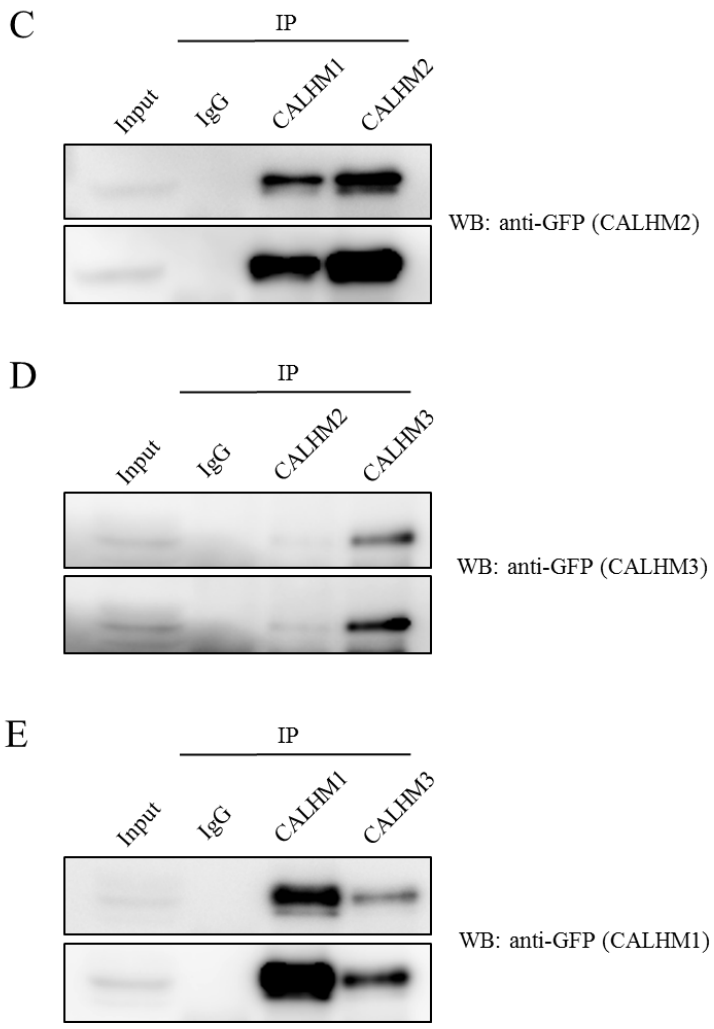

Figure 3. CALHM family interacts with each other.

(C) CALHM1 interacts with CALHM2 in HEK293T cells. The cells were transiently transfected with CALHM1-myc and CALHM2-GFP. Immunoprecipitated CALHM2-GFP was probed with anti-GFP antibody. (D) CALHM2 binds to CALHM3 in HEK293T cells. Cells were co-transfected

with CALHM2-myc and CALHM3-GFP. The immunoprecipitated CALHM2-myc and CALHM3-GFP with anti-CALHM2 and anti-CALHM3 antibodies were probed with anti-GFP antibody. (E) Interaction between CALHM1 and CALHM3 in HEK293T cells that co-expressing CALHM3-myc and CALHM1-GFP. Immunoprecipitated CALHM1-GFP was probed with anti-GFP antibody. Overexposure of the blot is shown in the lower panel, respectively.

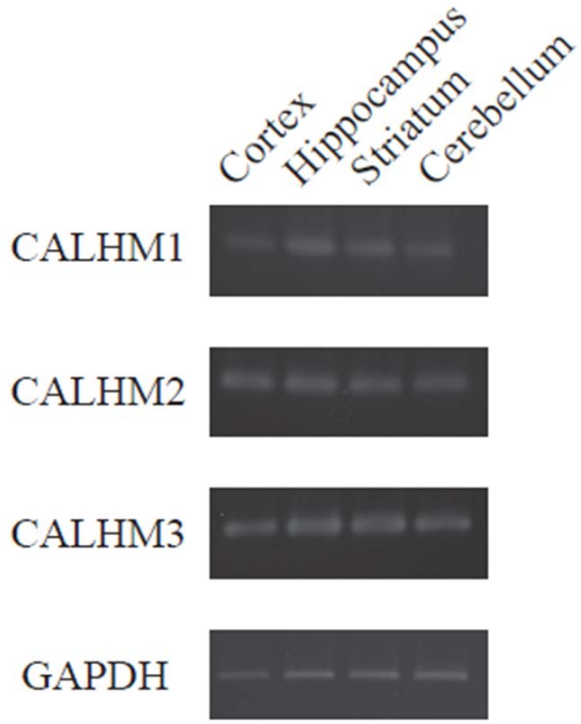

Figure 4. CALHM family subtypes are expressed in the brain.

The mRNA expressions of all CALHM family subtypes in various region of the brain were examined by RT-PCR. GAPDH was used as an internal control.

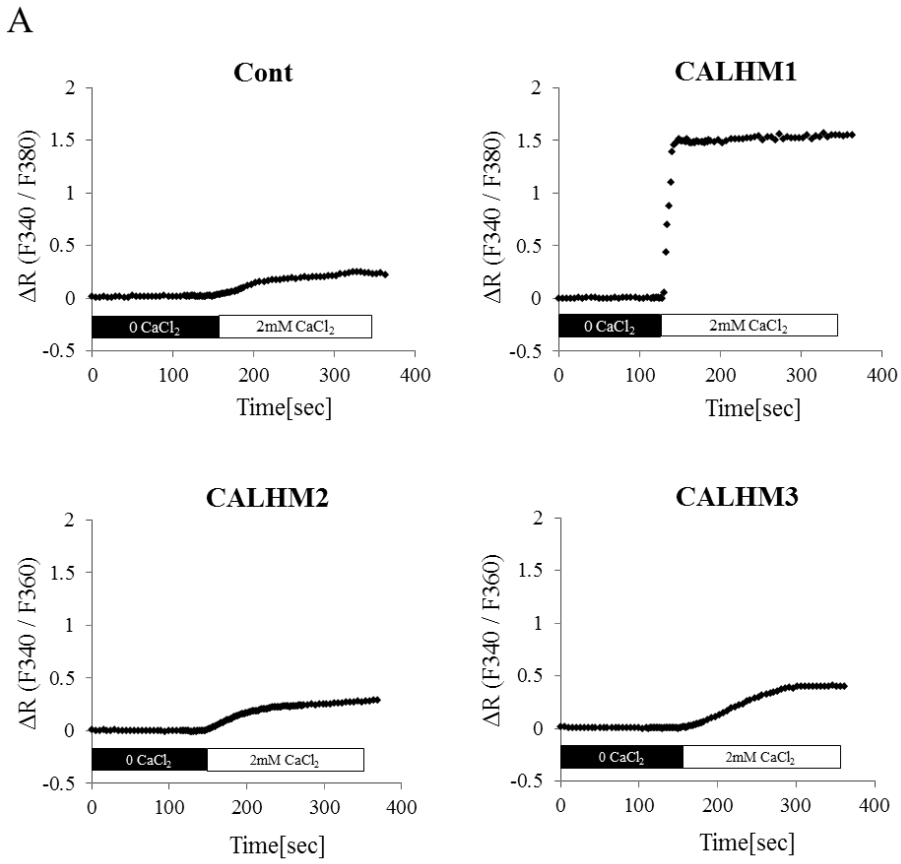

Figure 5. CALHM1 and CALHM3 are Ca^{2+} leaky channels.

(A) Measurement of Ca^{2+} entry in HeLa cells expressing each CALHM family proteins in response to extracellular Ca^{2+} elevation. Change of intracellular Ca^{2+} levels (ΔR, delta fura-2 ratio 340/380) of HeLa cells expressing CALHM family proteins upon the external Ca^{2+} elevation from 0 mM (0 $CaCl_2$) to 2 mM (2mM $CaCl_2$). Representative data of three independent experiments are shown.

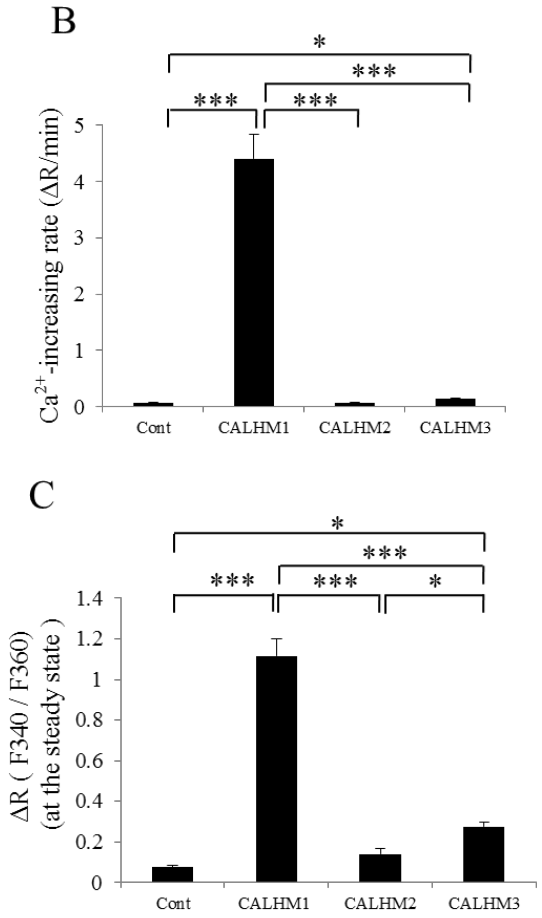

Figure 5. CALHM1 and CALHM3 are Ca^{2+} leaky channels.

(B) and (C) Quantifications of Ca^{2+}-increasing rate (ΔR/min) and the maximal $[Ca^{2+}]_i$ at the steady state (ΔR). The results are shown as the mean ± S.E. of at least three independent experiments. The total cell numbers were 122 (control), 120 (CALHM1), 58 (CALHM2), and 144 (CALHM3) cells, respectively. *, $p < 0.05$; ***, $p < 0.001$.

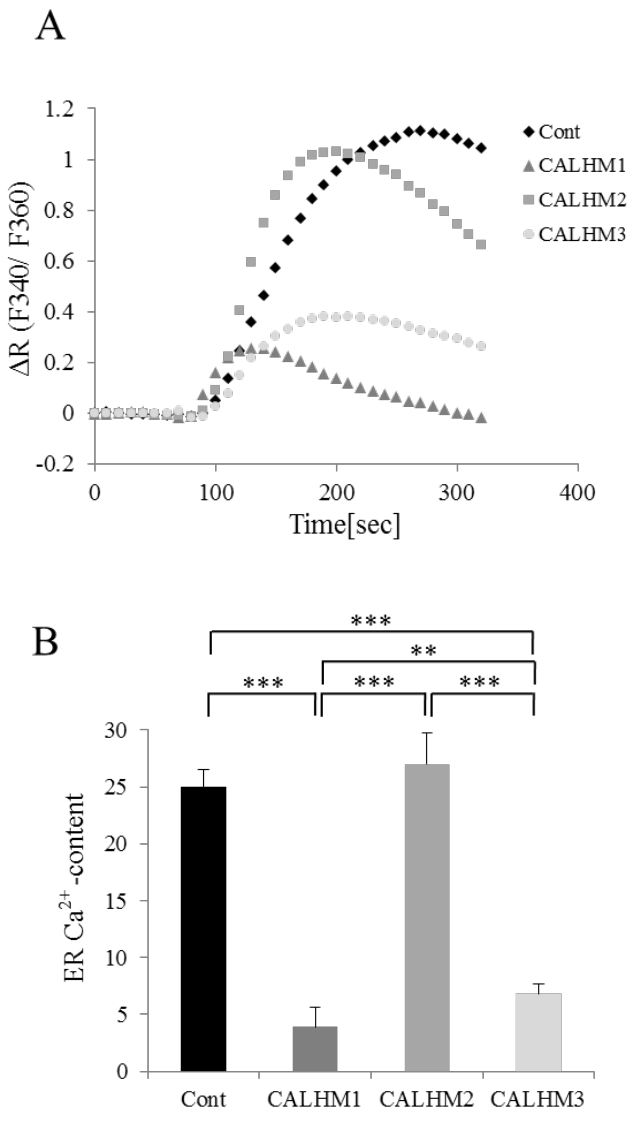

Figure 6. Overexpression of CALHM1 and CALHM3 decreases ER Ca^{2+} content of HeLa cells.

(A) Change of $[Ca^{2+}]_i$ in HeLa cells expressing CALHM family proteins upon CPA stimulation under Ca^{2+}-free condition. The experiments were

performed at least three times and the representative results are shown. (B) ER Ca^{2+} contents of HeLa cells expressing CALHM family proteins. Ca^{2+} -contents were expressed as area under curve (AUC) of Fura-2 ratio change. The results are shown as the mean ± S.E. of at least three independent experiments. The total number of cells were 91 (control), 41 (CALHM1), 36 (CALHM2), and 89 (CALHM3) cells, respectively. **, $p < 0.01$; ***, $p < 0.001$.

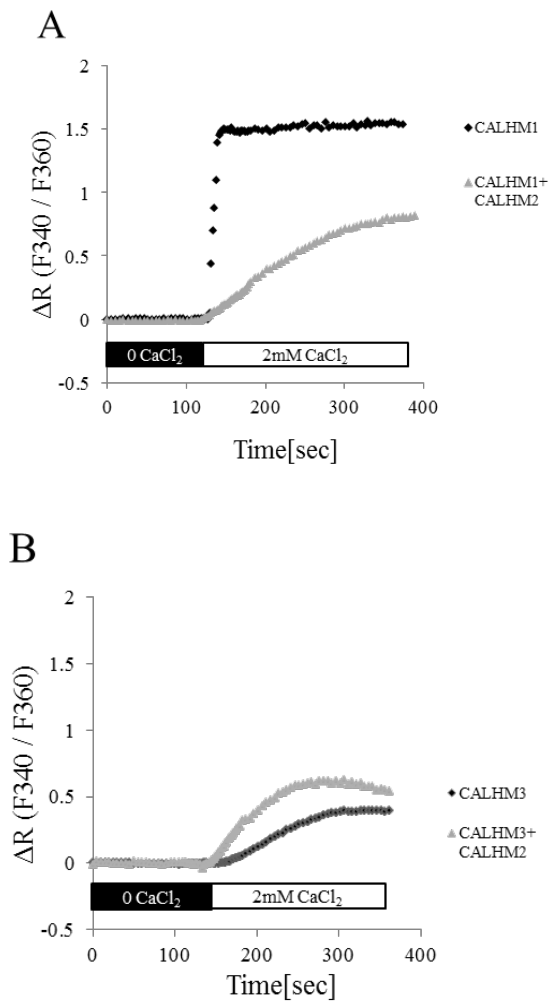

Figure 7. CALHM2 regulates CALHM1 and CALHM3 Ca^{2+} channel activity.

(A) and (B) Ca^{2+} entry of HeLa cells transfected with CALHM1 or CALHM3 and co-transfected with CALHM2. The representative changes of $[Ca^{2+}]_i$ upon external Ca^{2+} elevation from 0 mM (0 $CaCl_2$) to 2 mM (2mM $CaCl_2$) were shown (ΔR: delta change of fura-2 ratio of 340/380).

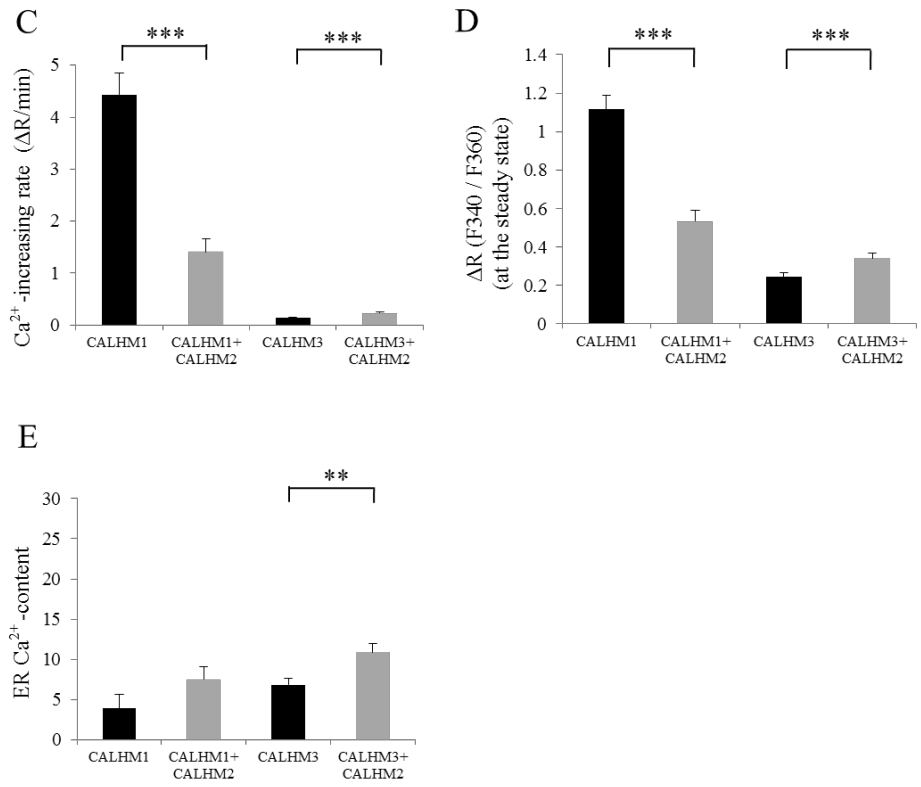

Figure 7. CALHM2 regulates CALHM1 and CALHM3 Ca^{2+} channel activity.

(C) and (D) Quantifications of Ca^{2+} -increasing rate (ΔR/min) and the maximal $[Ca^{2+}]_i$ at the steady state (ΔR). The total number of cells were 120 (CALHM1), 48 (CALHM1 and CALHM2), 143 (CALHM3) and 151 (CALHM3 + CALHM2) cells, respectively. (E) ER Ca^{2+}-contents of CALHM1 or CALHM3-single expressing cells and CALHM2 co-expressing cells with CALHM1 or CALHM3. ER Ca^{2+} content was calculated by area

under curve (AUC) of CPA-induced fura-2 ratio change in the absence of the external Ca^{2+}. The total number of cells were 41 (CALHM1), 54 (CALHM1 + CALHM2), 89 (CALHM3) and 81 (CALHM3 + CALHM2) cells, respectively. All results are shown as the mean ± S.E. of at least three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

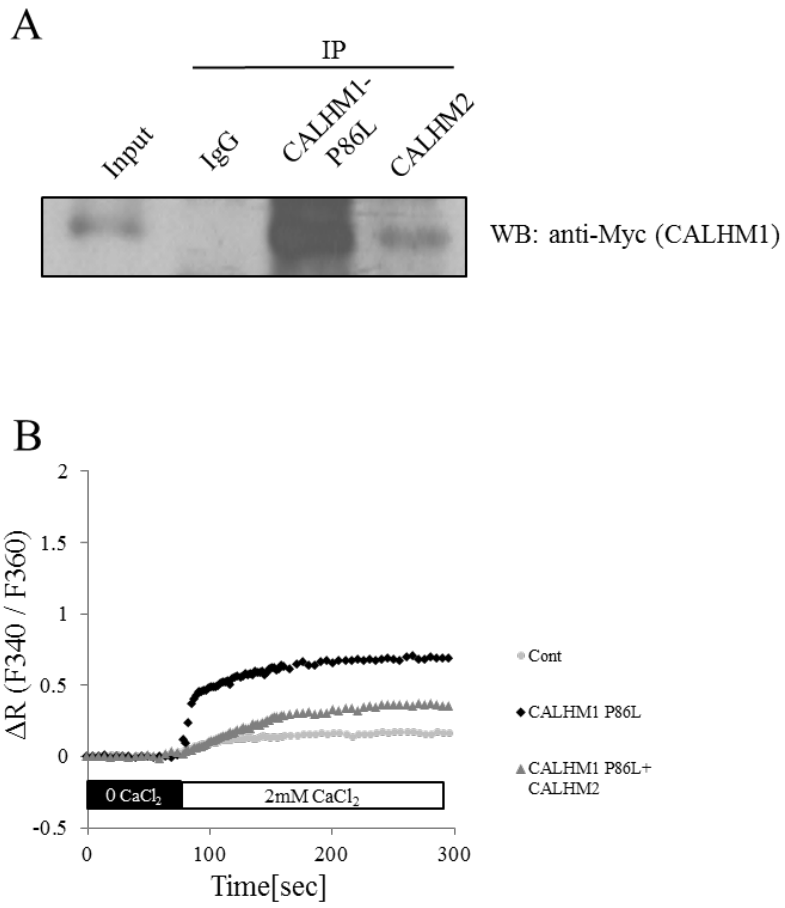

Figure 8. CALHM2 interacts with CALHM1-P86L and modulates channel activity of CALHM1-P86L.

(A) Interaction of CALHM1-P86L with CALHM2 in HEK293T cells. (B) Change of $[Ca^{2+}]_i$ in HeLa cells that express CALHM1-P86L and co-expression of CALHM1-P86L with CALHM2 upon extracellular Ca^{2+} elevation. Representative data of three independent experiments are shown.

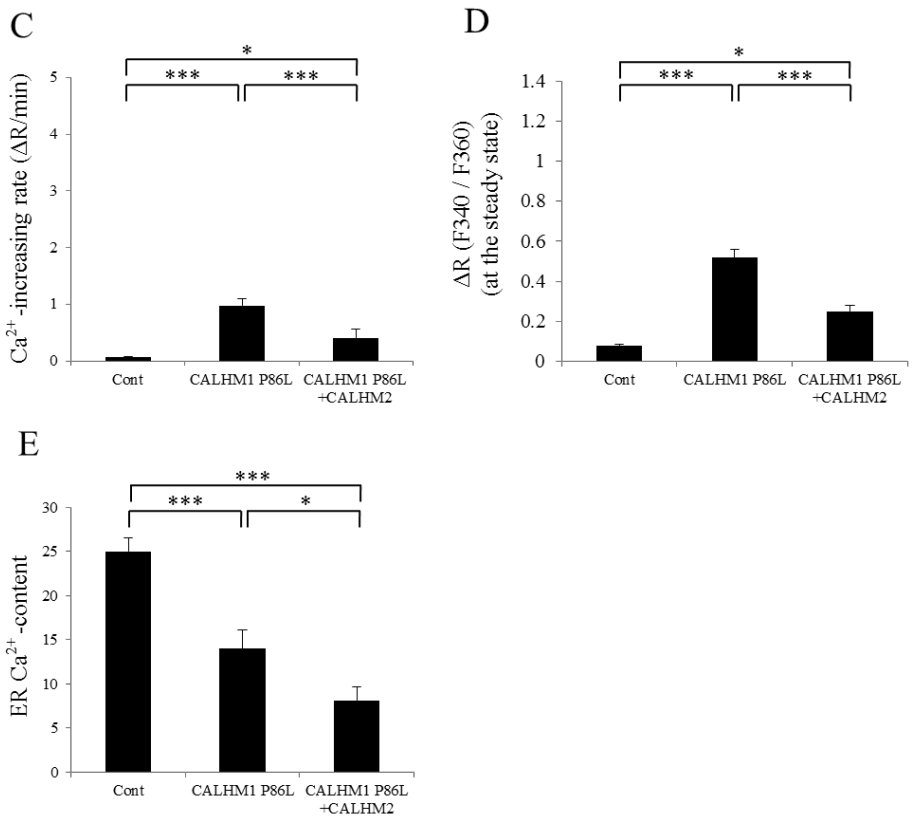

Figure 8. CALHM2 interacts with CALHM1-P86L and modulates channel activity of CALHM1-P86L.

(C) and (D) Quantifications of Ca^{2+} -increasing rate (ΔR/min) and the maximal $[Ca^{2+}]_i$ at the steady state (ΔR). The total number of cells were 122 (control), 144 (CALHM1-P86L) and 97 (CALHM1-P86L + CALHM2) cells, respectively. (E) ER Ca^{2+} content of CALHM1-P86L expressing cells or CALHM1-P86L and CALHM2 co-expressing cells. Data were shown as AUC. The total number of cells were 81 (CALHM1-P86L) and 58 (CALHM1-P86L

+ CALHM2) cells, respectively. All results are shown as the mean ± S.E. of at least three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

DISCUSSION

Although CALHM2 and CALHM3 share sequence homology with CALHM1 and have transmembrane domains similar to CALHM1 (Hirokawa, Boon-Chieng et al. 1998; Dreses-Werringloer, Lambert et al. 2008), the precise nature of CALHM2 and CALHM3 has not been extensively analyzed yet. In this study, we first demonstrated that CALHM2 and CALHM3 are localized at the ER and the PM and expressed in various regions in the mouse brain as similar to CALHM1. CALHM3 had the leaky Ca^{2+} channel properties as a homomeric channel however CALHM2 didn't show the Ca^{2+} channel activities. We also found that CALHM subtypes constitute heteromeric channels and that their mutual interactions differently modulate the channel activity of CALMH1 at the PM: CALHM2 reduces the Ca^{2+} permeability of CALHM1, whereas it increases that of CALHM3. Thus, our findings demonstrated a previously uncharacterized feature of CALHM families as heteromeric Ca^{2+} channels.

It is interesting that homomeric CALHM2 channel is impermeable for Ca^{2+}, whereas homomeric CALHM1 or CALHM3 exhibit the Ca^{2+} permeability. It

is known that CALHM1 has a short sequence within the COOH terminus of the hydrophobic domain 2 and has homology with the selectivity filter of the NMDA receptor (Dreses-Werringloer, Lambert et al. 2008). Accordingly, the asparagine 72 residue of CALHM1 was assumed to correspond critical residue for ion selectivity and permeation of NMDAR NR2 subunit (Wollmuth and Sobolevsky 2004) and was shown to be a key determinant in control of cytosolic Ca^{2+} levels (Dreses-Werringloer, Lambert et al. 2008). Therefore, we hypothesized that the difference of the amino acid residue might explain Ca^{2+} impermeability of CALHM2, however the corresponded asparagine residue is conserved for both CALHM2 and CALHM3. Thus, the difference of selective filter does not explain the Ca^{2+} impermeability of CALHM2.

Alternative possibility is the difference of precise intracellular localization between CALHM2 and other subtypes. Although we revealed that CALHM2 showed a similar pattern of intracellular localization as CALHM1 and CALHM3 by immunocytochemistry, we found the difference in the detergent solubility of CALHM2 as compared to CALHM1 and CALHM3: CALHM2

showed lower solubility for non-ionic detergent such as NP-40 and Triton X-100 than CALHM1 and CALHM3 (our personal observation), suggesting the distinct localization of CALHM2 in the lipid microdomain called “raft”. It is known that membrane rafts affect various ion channels by regulating the gating kinetics and the trafficking and surface expression (Dart 2010). Therefore, raft resident proteins and/or raft-enriched lipids such as cholesterol and sphingolipids might directly or indirectly exert on the Ca^{2+} permeability of homomeric CALHM2 channels.

In addition, we showed that CALHM2 regulates CALHM1 and CALHM3 channel activities in heteromeric channels: CALHM1/CALHM2 heteromeric channel has a lower Ca^{2+} permeability than CALHM1 homomeric channel, whereas CALHM2/CALHM3 heteromeric channel showed higher Ca^{2+} permeability than CALHM3 homomeric channel. Besides change of the channel pore properties by heteromerization, heteromerization of CALHM subtypes might also control their distinct localization within the PM, resulting in modulation of the channel activities.

The mutant form of CALHM1, CALHM1-P86L, was proposed to be an AD

risk factor (Dreses-Werringloer, Lambert et al. 2008; Boada, Antunez et al. 2010; Cui, Zheng et al. 2010), however its association with AD is still controversial (Inoue, Tanaka et al. 2010; Shibata, Kuerban et al. 2010; Feher, Juhasz et al. 2011). CALHM-P86L has reduced Ca^{2+} permeability (Dreses-Werringloer, Lambert et al. 2008) and shows slower cytosolic- and mitochondrial Ca^{2+} transients than those generated by CALHM1 (Moreno-Ortega, Ruiz-Nuno et al. 2010). In addition, CALHM-P86L was shown to increase the Aβ production (Dreses-Werringloer, Lambert et al. 2008). We examined whether CALHM2 regulates the CALHM1-P86L channel activity in a different way from wild-type CALHM1, but CALHM2 similarly interacted with CALHM1-P86L and inhibited the CALHM1-P86L channel activity. Therefore, the difference in the sensitivity of CALHM1-P86L for CALHM2 would not be directly associated with AD risk. However, because CALHM2 expression further decreases the Ca^{2+} permeability of CALHM1-P86L that has the reduced Ca^{2+} permeability as compared to wild-type CALHM1, the CALHM2 interaction with CALHM1-P86L might further promote the Aβ production in the brain.

It is known that ER stress triggers unfolded protein response (UPR) to protect the ER, but prolonged ER stress ultimately leads to cell death (Doyle, Kennedy et al. 2011; Stefani, Wright et al. 2012). Disturbed Ca^{2+} homeostasis within the ER is one of the causes that trigger ER stress (Lindholm, Wootz et al. 2006; Doyle, Kennedy et al. 2011) and has been thought to be related with the pathogenesis of some neuronal disease such as Parkinson's disease and AD (Katayama, Imaizumi et al. 2004; Lindholm, Wootz et al. 2006; Cali, Ottolini et al. 2011; Stefani, Wright et al. 2012). For example, many AD-related PS mutations affect their ER Ca^{2+} leak function, leading to the Ca^{2+} overload within the ER (Tu, Nelson et al. 2006; Supnet and Bezprozvanny 2010; Supnet and Bezprozvanny 2011).

Recently, it was reported that CALHM1 decreases Ca^{2+}-contents within the ER, which causes ER stress (Gallego-Sandin, Alonso et al. 2011). Since CALHM3 overexpression decreased Ca^{2+} stores within the ER, CALHM3 expression might also induce ER stress. In addition, we also showed that heteromelization of CAHLM3 with CALHM2 modulates the Ca^{2+} contents within the ER. Because the balance between Ca^{2+} influx and Ca^{2+} efflux from

the ER is important for determining the Ca^{2+} store size, the ratio change of hetero- and homomers of CALHM1 in the brain would affect Ca^{2+} store size and the subsequent ER stress. Further study, e.g. expression level of each CALHM subtypes in distinct neurons and regulation of their expression profile of CALHM family proteins in the pathological conditions of the brain will be an interesting issue to reveal as the physiological role of the heteromeric CALHM channels in the brain.

In summary, CALHM family proteins interact with each other and constitute heteromeric Ca^{2+} channels. Through these interactions, CALHM2 regulates channel activities of CALHM1 and CALHM3 and affects the intracellular and the ER Ca^{2+} concentration. We propose that regulation of Ca^{2+} signaling by heteromeric CALHM family proteins may provide new perspective in Ca^{2+} homeostasis in the brain.

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초록

칼슘이온은 세포의 생존에 필수적인 물질로서 세포는 칼슘항상성을 유지하며 다양한 기능을 수행하고 있다. 특히 신경세포 내에서 칼슘은 세포막의 흥분성, 시냅스 전달과정 등과 같은 주요한 기능을 조절하는데 중요한 역할을 하고, 칼슘 항상성의 저해는 알츠하이머 병과 같은 신경퇴행성 질환과 관련되어 있음이 보고되고 있다.

Calcium homeostasis modulator 1 (CALHM1)은 leaky 칼슘 채널로서, 주로 소포체 막에 존재하며 세포막에도 일부 존재하면서 세포 내의 칼슘 농도를 조절한다. CALHM1 의 돌연변이형인 CALHM1-P86L 은 알츠하이머 병의 원인 유전자로서 처음 보고되었지만 그 관련성에 대해서는 상반되는 결과들이 발표되고 있다.

또 다른 CALHM family 단백질로는 CALHM2 와 CALHM3 가 알려져있는데, 이들의 기능에 대해서는 거의 보고된 바가 없다. 하지만 CALHM family 단백질들은 서로 비슷한 아미노산 서열을 가지고 있으며 구조적으로도 3 개 또는 4 개의 막 통과 부분을 가지는 유사성을 보이기 때문에 본 연구에서는 전체

CALHM family 단백질이 칼슘 채널로서 작용할 수 있는 가능성과 이들간의 상호작용에 주목하였다.

그 결과 세포 내에서 CALHM family 단백질들간의 상호작용을 확인하였고, CALHM3 는 CALHM1 과 유사한 방법으로 세포 내에서 leaky 칼슘 채널로서 작용하는 것을 처음으로 관찰하였다. CALHM2 는 그 자체로는 채널로서의 기능을 보이지 않았지만 CALHM1 또는 CALHM3 와 함께 발현시켰을 경우에는 이들 자체의 채널기능을 변화시켰다.

이러한 결과들을 통해 본 연구는 칼슘 신호전달 과정의 새로운 메커니즘을 제시하였으며 CALHM family 단백질들이 실재 뇌에서 heteromeric 채널로서 세포 내 칼슘 항상성을 조절하는데 기여할 수 있는 가능성을 보여주었다.

주요어: 칼슘 항상성, CALHM (Calcium homeostasis modulator) family, 알츠하이머 병, 칼슘채널

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